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Early Identification of Stable Transformation Events by Combined Use of Antibiotic Selection and Vital Detection of Green Fluorescent Protein (GFP) in Carrot (*Daucus carota* L.) Callus

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Abstract

Genetic transformation is a useful technique to complement conventional breeding in crop improvement. Although carrot has been a model organism for *in vitro* embryogenesis study, genetic transformation of carrot is still lengthy and labor intensive. An efficient transformation and detection system is desirable. Direct infection of *Agrobacterium* to carrot calli has provided an easy way for carrot genetic transformation. To improve the efficiency of antibiotic selection in this method, we report the combined use of an improved green-fluorescent protein, referred to as smGFP, to establish a versatile selection method for carrot callus transformation system. By combining antibiotic selection with the bright fluorescence observed in the callus tissue, we were able to easily identify stable transformants in early stage of the transformation process. In addition to the GFP expression of the callus cells, the transgenic nature of callus cells was confirmed with Southern and Western analysis. We found we can link the simplicity of carrot-callus-cell transformation, early detection of stable transformants with antibiotic selection, visualization of GFP fluorescence, and molecular analysis (Southern and Western) of callus tissue (non-photosynthetic tissue) to provide a more efficient way in identifying stable transformants at early stage of carrot transformation.

Key words: *Agrobacterium tumefaciens*, antibiotic selection, *Daucus carota*, genetic transformation, reporter gene, stable transformed transgene

INTRODUCTION

Carrots represent a main source of vitamin A and fiber for human nutrition (Simon 1997; Horvitz *et al.* 2004). According to the annual report from Food and Agriculture Organization of the United Nations, more than 19 million metric tons of carrots were produced worldwide in 2005 (http://www.fao.org). Because of its central nutritional role in human health, improving the quality and yield of this major vegetable crop is important for improving its agronomic value. Conventional breeding has greatly contributed to the enhancement of carrot traits, such as the enrichment of β-carotene in carrot roots (Simon *et al.* 1989). However, gene transfer by genetic transformation can provide an important complementary technology to improve carrot quality and productivity (Gilbert *et al.*

1996; Chen and Punja 2002). It also serves as a useful tool for forward and reverse genetic study in carrot (Tang *et al*. 1999).

Several carrot genetic transformation systems have been reported in recent years, all depending solely on antibiotic selection to discriminate transformation events (Scott and Draper 1987; Thomas *et al*. 1989; Wurtele and Bulka 1989; Hardegger and Sturm 1998). Among those, the use of *Agrobacterium* to directly infect carrot callus cells has provided a simple and easy way for carrot transformation (Wurtele and Bulka 1989; Yau 2001). However, by using antibiotic selection solely in this method, mixtures of transformed and non-transformed cells in a surviving callus clump were often observed (Yau 2001). This might be due to cross-protection of transformed cells to non-transformed cells which allowed the non-transformed cells to escape from antibiotic selection. The cross-protection phenomenon was also reported in other plant transformation (Park *et al*. 1998). Transformation systems that do not allow for early identification of truly transformed plants are labor intensive and time consuming. Therefore, an early identification of stable and truly transformed callus to eliminate escapees so that only the truly transformed plants will be transferred to soil is important.

To ensure early identification of transformed cells, a stringent selection marker system must be combined with a readily visualized, preferably non-destructive reporter system. The combination of antibiotic selection with green fluorescent protein detection has been successful for rice (Vain *et al*. 1998) and legume transformations (Li *et al*. 1994; Cho and Widholm 2002). However, there is no such study for carrot callus transformation.

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has got a fluorescent ability to emit green light and can be directly expressed and visualized in the absence of other *Aequorea* proteins or any additional substrates or co-factors in a foreign organism (Chalfie *et al*. 1994; Haseloff and Amos 1995). GFP also has the advantage of lack of cell disruption that makes it an ideal selectable marker for gene expression analysis. Therefore, GFP was chosen as the visual reporter in this study. Although to our best knowledge, there are two papers published with carrot transformation with green fluorescent protein (Hibberd

et al. 1998; Baranski *et al*. 2006); however, one is dealing with GFP transient expression (not stable expression) by bombardment and the other one is using *Agrobacterium rhizogenes* (leading GFP expressing in adventitious roots) for transformation. No evaluation on GFP in carrot callus transformation was ever reported. In this study we focus on: (1) Can an improved GFP be expressed well in carrot callus? (2) Are these calli obtained from combined kanamycin and visual selections stably transformed, and can they be confirmed with Southern analysis on the putative transgenic callus?

MATERIALS AND METHODS

Plant materials

Carrot (*Daucus carota* L.) line B493 (Simon *et al*. 1990) was used for callus production and genetic transformation. Seeds were sterilized with 70% (v/v) ethanol for 2 minutes, followed by 5% (w/v) sodium hypochlorite (NaOCl) containing 0.02% (w/v) Triton x-100 (t-octylphenoxypoly-ethoxyethanol) (Sigma, St. Louis, MO, USA) for 15 minutes, and were rinsed 5 times with sterile deionized water from a Milli-Q UF Plus Water System™ (Millipore Corporation, Bedford, MA, USA). These seeds were then placed on freshly prepared MS regeneration medium, MS salts (Murashige and Skoog 1962) supplemented with 1 g mL^{-1} thiamine (vitamin B$_1$; Sigma), 0.1% (w/v) myo-inositol (Sigma), 3% (w/v) sucrose, and 1% (w/v) agar; pH 5.8, and placed under fluorescent lights (25 µmol m^{-2} s^{-1}) to induce seed germination.

Callus induction from hypocotyl tissue

Seedlings were grown under fluorescent lights (25 µmol m^{-2} s^{-1}) until 5 cm long, and hypocotyls were excised for use in callus induction. The hypocotyls were cut into 0.5 cm pieces, placed on 35 mL 1% agar-solidified callus-induction medium MSI (MS regeneration medium (see above) supplemented with 1.0 µg mL^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma) and 21.5 µg L^{-1} kinetin (Aldrich, Milwaukee, WI, USA)), and placed in the dark at room temperature. The resulting callus was subcultured every 3 weeks for four months

and then used for transformation.

Bacterial strain, plasmid vector, transformation and selection

Agrobacterium tumefaciens strain LBA4404 (Hoekema *et al.* 1983) bearing the binary vector pBIN-35S smGFP (Fig.1; Davis and Vierstra 1998; Shigaki *et al.* 2005) was used for transformation. The improved GFP (smGFP) used is a soluble version derived from the modified GFP designed for use in plants (Haseloff *et al.* 1997; Stewart 2001). A modification of the Wurtele and Bulka (1989) transformation method was used for carrot-callus-cell transformation. Briefly, *A. tumefaciens* was grown in 0.8% (w/v) Bacto Nutrient Broth solution (Difco Laboratories, Detroit, MI, USA) supplemented with 50 µg mL^{-1} kanamycin (Sigma) and 50 µL of this culture was directly added to B493 callus and mixed gently with a pair of sterile forceps. After 20 hours of co-cultivation in darkness, the callus/*Agrobacterium* mixture was evenly distributed on a sterile 7-cm diameter Whatman #1 filter paper overlying on 35-mL solidified selection medium (MS callus induction medium + 300 µg mL^{-1} kanamycin (Sigma) + 250 µg mL^{-1} cefotaxime, sodium salt (Sigma) to suppress bacterial growth. Calli were subcultured on selection medium every 3 weeks.

Genomic DNA extraction and Southern hybridization analysis

Southern-blot analysis was carried out according to the standard protocol of Sambrook *et al.* (1989). Putatively transformed callus was collected and lyophilized for two days. Total genomic DNA was extracted in a 2-mL plastic disposable microcentrifuge tubes from approximately 25 to 30 mg lyophilized transgenic or non-transgenic carrot callus according to the method described by Fütterer *et al.* (1995). The final DNA pellet was dissolved in 100 µL autoclaved Milli-Q water for Southern-blot analysis, or further diluted for PCR amplification. DNA was treated with RNase A and DNA concentration and purity were determined with a spectrophotometer.

A total of 5 µg of genomic DNA was digested with *Bbu* I (Promega, Madison, WI, USA), fractionated by

electrophoresis in a 1% (w/v) Tris Acetate EDTA (TAE) agarose gel, and transferred to a Zeta-probe™ nylon-blotting membrane (Bio-Rad Laboratories, Hercules, USA) by capillary transfer for 12 hours. After soaking in 2×SSC buffer for 10 minutes, the membrane was dried and baked at 80°C for 1 hour in a vacuum oven.

Two probes were prepared for DNA hybridization. (1) Probe for *npt*II gene hybridization: a partial *npt*II gene fragment was amplified from pBIN 35S-smGFP by PCR using oligmers NPT-35: 5´-CGT TTC GCA TGA TTG AAC AA-3´ and NPT-39: 5´-CCG CTC AGA AGA ACT CGT CA-3´ as primers. (2) Probe for *smGFP* gene hybridization: *smGFP* gene fragment was amplified from pBIN 35S-smGFP by PCR using oligmers degG3: 5´-GGA TCC GCT GGC GCC ACC ATG AGT AAA GGA GAA G-3´ and degG5: 5´-GAG CTC CTA CCC GGG TTT GTA TAG TTC ATC C-3´ (Davis and Vierstra 1996) as primers. Primers were synthesized by GibcoBRL company (Carlsbad, CA, USA). Primers were desalted before use for PCR amplification. PCR conditions were the same as that described later. The resulting PCR product was gel-purified using a GENECLEAN® Kit from Bio101® Systems (MP Biomedicals, Irvine, USA), and labeled with ^{32}P-dCTP using a DECAprime II™ Random Priming Labeling Kit (Ambion, Austin, Texas, USA).

Hybridization was performed at 42°C overnight in hybridization buffer (38% formamide, 1 mM EDTA, 0.25 M sodium phosphate, 7% SDS), followed by washing with 0.2×SSC + 0.25% SDS at 50°C for 20 minutes. The blot was then exposed to a Kodak BioMax MR film (Kodak, Rochester, New York, USA) for 3 days at -80°C and developed.

Probe for *npt*II gene was stripped from the blot by boiling the blot in 500 mL 0.1×SSC + 0.5% SDS solution at 95°C for 30 min, and rinsed with 2×SSC. The stripped blot was reused for smGFP probe hybridization.

PCR amplification of *smGFP* gene from total carrot-callus DNA

The integrated smGFP sequences from putative transformants were detected by PCR. The extracted DNA and the oligomers (1) degG3: 5´-GGA TCC GCT GGC GCC ACC ATG AGT AAA GGA GAA G-3´ and (2) degG5: 5´-GAG CTC CTA CCC GGG TTT GTA

TAG TTC ATC C-3′ (Davis and Vierstra, 1996) were used for PCR amplification. Each PCR tube contained 2 µL of the total DNA solution, 1×PCR buffer, MgCl$_2$ (15 mM), dNTP mixture (0.5 mM), 1 U *AmpliTaq*™ DNA polymerase (Perkin Elmer, Wellesley, MA, USA), 1 mM of each primer and water for a total volume of 20 µL. Thermocycle schedule was 1 cycle at 94°C for 2 min, 35 cycles at 94°C (30 s), 56°C (1 min) and 72°C (2 min), then finally 1 cycle at 72°C for 10 min. All PCR were performed on a *GeneAmp* PCR System 9600 machine (Perkin Elmer). The amplification products were analyzed in 1% TAE agarose gels stained with ethidium bromide.

Immunological techniques

To check for expression of smGFP in the transformed callus cells, Western blotting was performed. For protein extraction, 100 mg of putatively transformed callus tissue was purified by first grinding callus in liquid nitrogen and then resuspending the powdered tissue in 50 mM Tris pH 7.5, 100 mM NaCl, 10% glycerol. Proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto a polyvinylidene difluoride membrane (Sambrook *et al*. 1989). Anti-smGFP serum was produced in a New Zealand White rabbit with highly purified, recombinant smGFP as the antigen (Davis and Vierstra 1998), and used to probe the blot. The antigen-antibody complex was detected with alkaline phosphatase-labeled goat anti-rabbit immunoglobulins and the substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate according to manufacturer´s instructions (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA).

Detection of GFP fluorescence in transformed carrot callus cells

Carrot callus was vortexed in water to isolate individual cells, this slurry was applied to a glass slide, and mounted with a coverslip. Microscopic examination of these living cells was with an Olympus BX60 microscope imaged through brightfield objectives or through an FITC filter cube (excitation filter 470-490 nm; dichroic mirror 505 nm; emission filter 515-550 nm) using a Sensys cooled charge-coupled-device camera with an exposure time of 1 s.

RESULTS

Seed germination and callus induction

The germination rate of carrot seeds sterilized with ethanol and NaOCl was greater than 98%. Seeds germinated in about 7 days. No contaminated seeds or seedlings were observed. Healthy yellow calli were observed under a dissecting microscope from hypocotyl segments in 5 days.

Callus transformation and recovery of the transformed callus lines

After transformation, plates were placed in the dark for selection for 5 weeks, and then 3 weeks under light. Three weeks of selection in light accelerated the growth of the transformed calli (data not shown), and resulted in a clear contrast between brown dead cells and yellowish transformed cells after 8 weeks on selection medium (Fig.2).
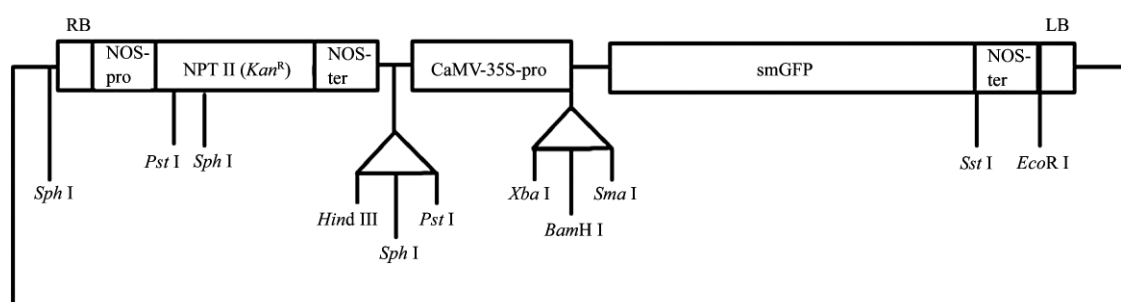

Fig. 1 The construct of binary vector pBIN 35S-smGFP; *npt*II gene and smGFP gene were shown.

The number of putatively transformed discrete callus varied from 1 to 11 per plate and sizes varied. Putatively transformed callus was subcultured to fresh selection medium every 3 weeks for further growth to allow for tissue to be used for PCR and Southern analysis. It took 14 weeks from start of selection for the first visible callus clump to grow to 0.7 cm in diameter. Control calli (without transformation) did not grow on the selection medium suggesting that growing calli was transformed.

Total genomic DNA extraction and PCR of antibiotic-resistant carrot calli

Seven individual antibiotic resistant discrete calli were selected randomly for preliminary PCR screening of stable transformation. We observed the expected 700 bp PCR product from all 7 transformed calli and the positive control sample (smGFP vector), and there was no PCR product observed when non-transformed calli genomic DNA was used for amplification (Fig.3-A).

Southern blot analysis for putatively transformed calli

Genomic DNA of a non-transformed (negative control), three transformed calli, and a positive control (vector
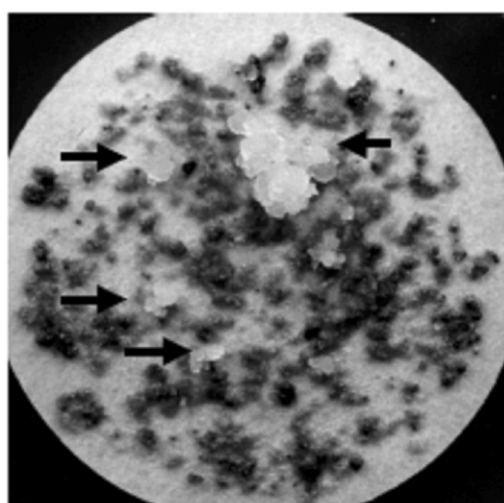
smGFP) was extracted and digested overnight in 37°C with *Bbu* I restriction enzyme (isoschizomer of *Sph* I; Fig.1), and used for Southern analysis with *npt*II gene derived NPT-35, NPT-39 amplified frament as a probe. As expected, both 1.0 kb and 1.6 kb bands were observed in the positive control (vector smGFP) lane (Fig.1, Fig.3-B). The non-transformed individual callus exhibited no hybridization signal while the transformed calli exhibited the 1.6 kb band and other larger bands (Fig.3-B). This confirms that the transgene was integrated into the carrot genome. The 1.0 kb band which appeared in positive control (vector smGFP) (Fig.3-B, lane 5) was not observed in the transformed callus total genomic DNA (Fig.3-B, lanes 1 and 2); as this *Bbu* I site was lost upon integration of the T-DNA insert into carrot genome. Larger bands were observed
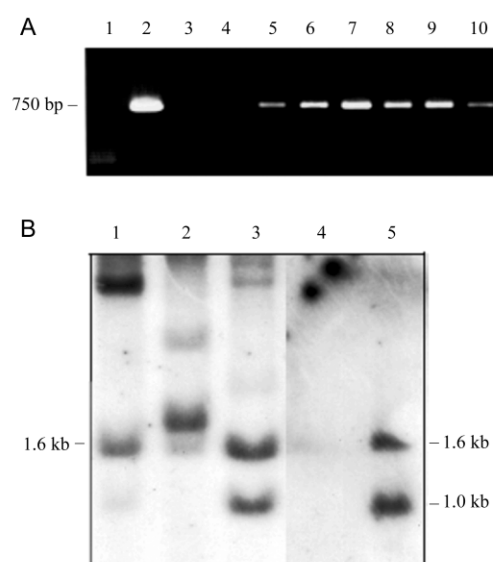

Fig. 3 Confirmation of putatively transformed carrot callus by PCR and Southern blotting. Six putatively transformed calli and two non-transformed calli were used for DNA extraction and PCR amplification. A, PCR products amplified from total DNA of putatively smGFP-transformed discrete calli and negative controls. Lane 1, no DNA (negative control); lane 2, positive control (smGFP vector); lanes 3 and 4, negative control (non-transformants); lanes 5-10, independent yellow putatively smGFP-transformed calli. Arrow marks PCR product (750 bp) from GFP-gene-specific primer amplification. B, three DNA samples were randomly selected from the above (A) six putatively transformed calli to perform Southern blotting. One DNA sample from the non-transformed callus was used for negative control. An *npt*II probe was used for Southern hybridization analysis of carrot genomic DNA extracted from non-transformed callus clumps (controls) and putatively smGFP-transformed calli. Lanes 1-3, three independent putatively transformed calli were digested with *Bbu* I; lane 4, non-transformed callus (negative controls) digested with *Bbu* I; lane 5, positive control (smGFP digested with *Bbu* I).

Fig. 2 Putatively smGFP transformed carrot B493 callus clumps (arrows) growing on MSI medium supplemented with 300 μg mL^{-1} kanamycin.

for the transgenic calli in the autoradiograph due to *Bbu* I cleavage in T-DNA sequence and in carrot genomic DNA. Along with the 1.6 kb band, we also observed the 1.0 kb band in one putatively transformed callus (Fig.3-B, lane 3) suggesting that a *Bbu* I site at (or near) the flanking region of right border of the T-DNA after insertion or the possible presence of some non-integrated T-DNA. The probe from the blot was stripped, and the blot was reused to hybridize with probe for smGFP. Transgene signals were detected (data not shown). These two hybridization results suggested that intact T-DNA was transferred from right border to left border, not disrupted, and integrated into carrot genome. Callus without the 1.0 kb band in Southern blotting was then used to exam GFP production.

Western blotting

The same callus used in Fig.3-B lane 2 for Southern blotting was used for Western blotting. Antibody of smGFP was hybridized to a positive control smGFP protein (data not shown) and smGFP protein in the transgenic carrot callus (Fig.4-A, lane 2). One band (27 kD) with the expected molecular weight was observed. No band was observed from the non-transformed callus (Fig.4-A, lane 1).

Green fluorescence was observed from the transformed callus cells

smGFP fluorescence in transformed callus cells of carrot was observed (Fig.4-B III). No fluorescence was observed in the non-transgenic carrot callus (Fig.4-B IV), and little auto-fluorescence background was observed in the non-transformed carrot callus suspension cells (Fig.4-B IV).

DISCUSSION

Agrobacterium-mediated genetic transformation of plants is an attractive alternative to direct DNA delivery methods such as gene bombardment. The advantages of the *Agrobacterium* system include the characteristic insertion into the recipient genome of a discrete segment of DNA at a low copy number (Klee *et al*. 1987). This contrasts with the complex rearrangement and multiple integration events that are typical outcomes of biolistic
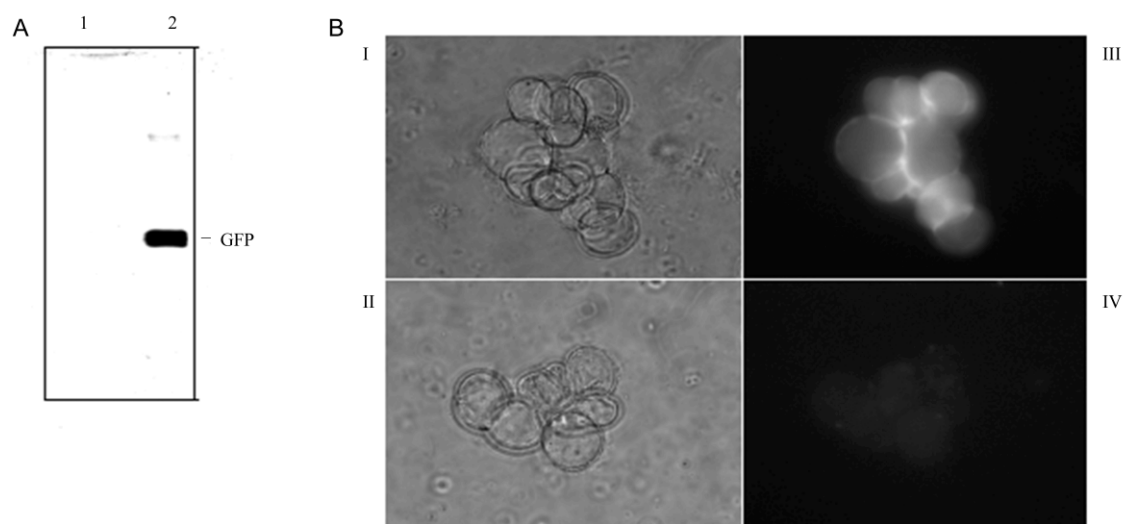

Fig. 4 Western blot and visual detection of GFP in transformed carrot. Putatively transformed callus used in Southern blotting analysis (Fig. 3-B, lane 2) was also used for western blotting analysis and visual detection. A, Western-blot analysis of proteins reacting with anti-smGFP serum. Lane 1, proteins extracted from non-transformed callus; lane 2, proteins extracted from smGFP-transformed callus. Arrow marks the GFP protein (27 kD). B, visual detection of smGFP expression in carrot callus cells. smGFP construct was transformed into carrot callus cells mediated by *Agrobacterium tumefaciens*, fluorescent cells were visualized and pictures were taken with a Sensys CCD camera magnification. The fluorescent cells were viewed by blue excitation (470-490 nm) and green emission (515-550 nm). I, smGFP-transformed callus cells under a white light; II, non-transformed (control) callus cells under a white light; III, smGFP-transformed callus cells under blue excitation; IV, non-transformed (control) callus cells under blue excitation.

delivery of DNA (Peng *et al*. 1995; Wu *et al*. 1998). An efficient gene transformation system by using *Agrobacterium* to direct infect carrot callus cells has been reported (Wurtele and Bulka 1989). However, by using antibiotic selection solely in this method, mixtures of transformed and non-transformed cells survived in a callus clump (Yau 2001). To improve the efficiency of selection, we report the combined use of antibiotic selection with an improved green-fluorescent protein, referred to as smGFP, to establish a versatile selection method for carrot callus cell transformation system.

We successfully expressed an improved *gfp* gene (smGFP) in the carrot (*Daucus carot*a L.) callus cells derived from seedling roots, and the combined use of kanamycin selection to allow for accurate, non-destructive events scoring of transgenes with small calli. The transformed callus cells exhibited bright fluorescence, comparable to that of the *Arabidopsis* and tobacco published elsewhere (Haseloff *et al*. 1997). No fluorescence was observed in untransformed callus cells. Little auto-fluorescence was observed in untransformed callus cells. These results suggest that GFP can be a good visual marker for using in carrot-callus transformation system. Fusion proteins between an exdogeous protein and GFP can be created and will be also useful for both carrot transformation and other biological research.

Early detection of a stable transformed transgene by Southern blot analysis using callus tissue was also incorporated in existing carrot-callus transformation system. Southern blot analysis using small amount of calli of putative transformants could help improve the efficiency of selecting of stable transformants in a carrot improvement program since confirmation of transformation can be made 14 weeks after initiation of transformation, in contrast evaluation after at least 8 months using leaf tissues regenerated from transgenic plants (Wurtele and Bulka 1989).

The results from this study indicated that the improved green fluorescent protein (smGFP) (Davis and Vierstra 1998) expressed well in carrot callus and found bright, fluorescent cells. The combined use of kanamycin and the GFP selections have allowed us for accurate, non-destructive events scoring of transgenes with small calli. The transgenic nature of callus cells was confirmed with Southern and Western analysis as well.

By combining the advantages of (1) easy induction of callus from carrot seedling hypocotyls, (2) the simplicity of callus infection with *Agrobacterium* for transformation, (3) use of GFP as a non-destructive reporter protein, and (4) early detection of stable transgenic cells in callus (not differentiated tissues) as material for Southern blot analysis, a more efficient way for gene transfer to carrot can be expected. Furthermore, the non-destructive procedure for GFP selection will allow for the possibility to save many putatively transformed calli for regeneration (Kaeppler *et al*. 2000).

Since the regeneration of carrot plants from callus have been routine, this research focused only on the study of efficient selection of transformed callus from callus transformation. We believe that smGFP will be a useful tool for carrot transformation and their biological studies.

Acknowledgements

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